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Research Article

Pollen metabarcoding of museum specimens and recently collected bumblebees (*Bombus*) indicates foraging shifts

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Abstract

Landscape changes, over time, lead to changes of floral resources available to pollinators, which in turn may result in the disappearance of ecologically specialized species. Here, we use pollen metabarcoding to infer historic and recent interactions between plants and bumblebees (*Bombus*). Bumblebees from Cuxhaven (Germany) were sampled from historical museum collections (1968/69) and in the field (2019). Pollen attached to their bodies was barcoded using multiple plant markers (ITS1, ITS2 and *trnL*-P6 loop). Our results show shifts in foraging habits between the historic and recent sampling periods, mostly determined by fewer Fabaceae interactions in 2019. The successful implementation of scalable molecular techniques for the analysis of historical pollen samples underscores the value of museum collections as a resource for biodiversity research. This study provides proof of concept of a comparative analysis of recent and historical pollination data. However, to ensure the robustness of our results, it is crucial to consider the broader methodology used. Our study found variation in the efficacy of the three plant barcoding markers. The ITS1 marker exhibited the highest species-level identification success, while the trnL-P6 loop demonstrated utility in amplifying degraded DNA across diverse plant families.

Key words: barcoding, *Bombus*, bumblebee, Cuxhaven, Hamburg, ITS1, ITS, ITS2, natural history collection, plant metabarcoding, pollen, *trnL*-F P6

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Introduction

The biodiversity of insects and especially pollinators is in rapid decline (Potts et al. 2010; Hallmann et al. 2017; Goulson 2019; Sánchez-Bayo and Wyckhuys 2019; Janzen and Hallwachs 2021), exacerbated by a negative feedback cycle of plants and pollinators (Biesmeijer et al. 2006; Thomann et al. 2013). This impacts the stability of the ecosystem services of pollination (Gallai et al. 2009; Bauer and Sue Wing 2016; IPBES 2016; Rhodes 2018). A major driver of pollinator loss is land-use change as a result of agricultural intensification and urbanization (Kleijn et al. 2009; Habel et al. 2019; Seibold et al. 2019; Vray et al. 2019; Suzuki-Ohno et al. 2020; Rollin et al. 2020; Raven and Wagner 2021; Dicks

et al. 2021; Köthe et al. 2023), which also affects the plant community composition. Other drivers include pesticide use, climate change and habitat degradation (Wagner 2020). Biomonitoring of pollinators and their pollination service is an important tool to track plant and insect population changes and to potentially counteract population loss through conservation measures (Breeze et al. 2021). Bumblebees (*Bombus* ssp.) are a suitable taxon for such analyses because they are easy to spot and can be found in large numbers in natural history collections.

Bumblebees are important pollinators with great ecological and environmental impact (Willmer et al. 1994; Luca and Vallejo-Marín 2013; Ogilvie and Thomson 2015; Sapir et al. 2017). They are tolerant to colder climates (Williams 1998; Hines 2008), able to navigate well in difficult environments (Vries et al. 2020) and are capable of buzz pollination (Luca and Vallejo-Marín 2013). The pollen foraging preference of bumblebees is guided by continuous assessment of the nutritional value of the pollen, such as lipid and protein content (Ruedenauer et al. 2016; Hendriksma et al. 2019; Vaudo et al. 2020). In contrast, bumblebees' nectar foraging is primarily directed towards high sugar concentrations (Konzmann and Lunau 2014). Most bumblebee species are generalist pollinators (polylectic), but their interspecific preferences can still differ markedly (Kleijn and Raemakers 2008; Vray et al. 2017; Timberlake et al. 2019). Consequently, focusing on multiple taxa in bumblebee conservation is required to ensure a high local pollination efficiency (Bommarco et al. 2012; Wood et al. 2021). In some bumblebee species the relationship between dietary focus and distribution decline is not as clear as in others (Connop et al. 2010), but, there are numerous examples of accelerated decline of bumblebee species with a narrow dietary range (Goulson et al. 2005; Goulson et al. 2008; Kleijn and Raemakers 2008; Wood et al. 2019). Species with a dietary focus on Fabaceae have been reported to be especially vulnerable (Goulson et al. 2005; Wood et al. 2021). Despite the benefits of bumblebee monitoring (Breeze et al. 2021), bumblebee distribution data collection has received little attention in the past, and still does, resulting in poorly harmonized datasets and spatial and temporal gaps (Graves et al. 2020; Cameron and Sadd 2020), exacerbated by some datasets being not open-access (King et al. 2019). One possible solution to close this knowledge gap in the future is to implement a monitoring scheme for pollinators, including bumblebees (Potts et al. 2021). Technical innovations, such as new camera technologies and genetic tools may help to implement efficient monitoring.

Metabarcoding is a promising approach for large-scale biomonitoring (Fahner et al. 2016; Elbrecht and Steinke 2018; Hardulak et al. 2020; Schwentner et al. 2021). However, improper setup of various experimental stages might affect the final results (Thomsen and Willerslev 2015), calling for strict quality control (Thalinger et al. 2021). Nonetheless, the technique provides powerful data to tackle biodiversity challenges (Beng et al. 2016; Barsoum et al. 2019; Liu et al. 2020). Metabarcoding cannot only be used to directly monitor the pollinators, but also to study their diet by analyzing their foraging preferences based on pollen remains (Grozinger and Zayed 2020). Pollen metabarcoding has shown to be equal or superior to microscopic studies (Smart et al. 2017; Macgregor et al. 2019; Campbell et al. 2020; Polling et al. 2021). Studies comparing observations of flower visitation with pollen metabarcoding also confirmed that pollen metabarcoding was able to recover a higher amount of plant-pollinator interactions (Pornon et al. 2017; Piko et al. 2021). In addition to

these benefits, metabarcoding museum collections allows a window into the past (Ewers-Saucedo et al. 2021).

The city of Cuxhaven (Lower-Saxony, Germany) and its surrounding areas have been in the focus of historic bumblebee studies (Wagner 1969), with specimens being deposited at the Leibniz Institute for the Analysis of Biodiversity Change (Hamburg, Germany). Reports of increasing urbanization by Wagner (1969) foreshadowed a bleak future for some of the 13 different non-parasitic bumblebee species detected at that time. We chose the same area to investigate whether molecular tools are able to identify shifts in foraging between the past and present, which is an important factor in bumblebee wellbeing and also affected by urbanization. Our investigation of shifts in flower-pollinator interactions focused on differences in the composition of pollen loads of bumblebees sampled in a previous study by Wagner (1969) and newly collected specimens in 2019 by using pollen metabarcoding on corbicula pollen and pollen sampled on the bodies of recent bumblebees. We also compared technical aspects, such as the efficacy of the *trnL*-P6 loop versus the ITS1 and ITS2 (from here on ITS1/2) plant DNA barcoding markers.

Materials and methods

Sampling

Bumblebees were collected in Cuxhaven (Germany: Lower Saxony) in proximity to the coastline (700 m) between the coordinates 53°51'36.3"N, 8°35'58.4"E and 53°52'55.4"N, 8°37'49.0"E, including the protected area Cuxhavener Küstenheiden (WDPA-ID: 329318, protected since 2004). The sampling sites varied between disturbed ground (suburbia), wet pasture, sandy scrubland and heath. Gardens, farmland (including pastures) and broadleaf forest were within 1 km radius. Sampling took place on the 2019-07-31 between 11 am and 4 pm in sunny weather. Permission to collect specimens and to enter the protected areas was granted by the municipal administration of Cuxhaven (Fachbereich 4: Naturschutzbehörde und Landwirtschaft). For sampling, we focused on female worker bees with visible pollen loads from as many different species as possible. We occasionally caught male bumblebees, which were determined after sampling, but were kept and analyzed alongside the female worker bees. To our best knowledge, no queen bees were caught. Bumblebees were directly caught in new, clean 50 ml centrifuge tubes (one specimen per tube) and then subsequently frozen overnight. We kept tubes upright, however, due to transport conditions, we cannot exclude minimal transfer of corbicula pollen to the bumblebee's body. Pollen samples were taken within 24h thereafter by removing the pollen from the corbicula (if available) and by dabbing the bumblebee's body with a toothpick covered in glycerol gelatin (except the hind leg). In 2019, we collected 120 specimens, of which 117 specimens were included in the following analysis.

Historic pollen samples from Cuxhaven were retrieved from the bumblebee collection of the Zoological Museum Hamburg (ZMH, Museum der Natur, Leibnitz Institute for the Analysis of Biodiversity Change, Hamburg). Female worker bumblebees were screened for pollen packages at their hind leg. We focused our efforts on samples collected by Rainer Wagner (Wagner 1969). The pollen packages were removed by precision tweezers (Dumont T5036). If the sample

quantity was sufficient, one of the pollen packages was not removed for subsequent analysis. Pollen taken from the body hairs of historic bumblebees did not result in any PCR amplicons, regardless of the marker used (data not shown). In total, we sampled 81 historical bumblebees. These samples represent the subset of bumblebees caught by Wagner (1969), which were deposited in the museum and had visible pollen packages at their hind legs.

Bombus identification

Bumblebees were identified independently by COI barcoding with DNA extracted from one hind leg, after pollen removal, and morphologically without disparities. Following the manufacturers' protocol, the BioSprint 96 DNA Blood Kit (Qiagen) was used for automated DNA extraction with a Biosprint 96 (Thermo-Fisher). PCR targeting the 658 bp mitochondrial COI barcoding fragment was done using the primers HCO2198-JJ and LCO1490-JJ (Astrin and Stüben 2008). PCRs were set up in volumes of 20 µl (2 µl DNA template, 0.8 µl of each primer (10 pmol/µl), 10 µl PCR Multiplex Mastermix (Qiagen, Hilden, Germany) and 6.4 µl H_2O). PCR was performed by two different cycling regimes: 15 cycles (denaturation for 35 s at 94 °C, step-down annealing at for 90 s at 55 °C with −1 °C per cycle, extension for 90 s at 72 °C) immediately followed by 25 cycles (denaturation for 25 s at 94 °C, annealing for 90 s at 45 °C, extension for 90 s at 72 °C). Sanger sequencing with the same primers was outsourced to BGI (Hongkong, China). Sequences were assembled and analyzed by Geneious 7.1.8 (Kearse et al. 2012). Voucher specimens are accessible (deposited in Zoological Museum Hamburg: ZMH844169–ZMH844289).

Pollen metabarcoding laboratory workflow

To avoid contamination, we treated surfaces, labware and plastic equipment with UV light and additionally with DNA AWAY (ROTH X996.2, Karlsruhe, Germany) before use. DNA extractions and the setup of PCRs were performed in a sterile flow cabinet. The DNA extraction and PCR protocols were tested for various parameters and robustness (Kolter and Gemeinholzer (2021a), Suppl. material 1). Approximately 20% of all sequenced PCRs were blanks (PCR template negative controls). Due to the potential risk of cross-contamination into low-DNA samples (1968/69), the decision was made to exclude positive extraction controls from the experiment.

The DNA extraction buffer was modified from Sellers et al. (2018), while the extraction protocol was modified from the NucleoMag DNA extraction kit (REF744400.4 Macherey-Nagel, Düren, Germany). Samples were homogenized by bead milling in 1.5 ml tubes with six 1.5 mm stainless steel (grade SAE 316L) balls for 2.5 minutes at 30 Hz (MM400 Retsch, Haan, Germany) and DNA was subsequently isolated with a downscaled magnetic bead extraction protocol (Suppl. material 2). DNA was eluted by adding 35 µl of pre-warmed buffered H_2O (5 mM Tris/HCl pH 8.5).

The ITS2 PCR protocol and sequencing strategy was previously described in Kolter and Gemeinholzer (2021a). Identical protocols were applied to ITS1, using the primers ITS-2plR1 and ITS-u1 (Cheng et al. 2016), modified by adding a TruSeq HT (Illumina) primer tail to enable tagging by a subsequent PCR

(Suppl. material 3). The *trnL*-P6 primers g and h by Taberlet et al. (2007) were modified in the same way. Due to their age, samples from 1968/69 were not amplified with the ITS1/2 markers, as initial trials revealed little success (data not shown). PCR was performed in individually tagged triplicates (Suppl. material 3). The paired-end 300 bp MiSeq sequencing run was performed on 1 ½ flow cells by LGC Genomics (Berlin, Germany).

Reference databases and bioinformatic analysis

Reference databases for ITS1/2 and *trnL*-P6 were generated from GenBank data files and filtered subsequently by a custom R script (Suppl. material 4). Reference database alignments were done with MAFFT and ITS regions were extracted using ITSx (Bengtsson-Palme et al. 2013; Katoh and Standley 2016).

The custom bioinformatic pipeline to analyze the MiSeq data used the R packages dada2 (featuring the DADA2 algorithm), vegan and ShortRead (Morgan et al. 2009; Callahan et al. 2016; R Core Team 2021; Oksanen et al. 2022). In contrast to ITS1/2, the read length of 300 bp was sufficient to always cover the whole *trnL*-P6 amplicon (< 160bp). To maximize the number of reads available for downstream analysis, the reverse (R2) sequencing reads were eliminated from the *trnL*-P6 workflow (Suppl. material 5). Amplicon sequence variant (ASV) generation by DADA2 was followed by multiple filtering steps. (Suppl. material 5). ASVs which could not be found in two out of three PCR replicates per sample were removed; this has been shown to minimize false positives and false negatives (Yang et al. 2021). The taxonomic assignment by SINTAX (Edgar 2016) was manually checked for plausibility. To address background noise-type contamination and more sporadic contamination types, a two-step process was implemented. First, ASVs whose sum of read count across all blanks surpasses 10% of their total read count across all samples were eliminated. Second, background contamination was mitigated by calculating the 90th percentile of read counts across all blank samples for each ASV, respectively, and subtracting this value from the read counts of the corresponding ASV in all other samples. Any resulting negative values were adjusted to zero. ASVs only found in blanks were ignored (Suppl. material 5). Sequentially, read numbers of ASVs which were identified to the same taxonomic entity by SINTAX were conflated. The read numbers were transformed into presence / absence data. Pollination network graphs were generated by the R package bipartite (Dormann et al. 2009). The bioinformatic R pipeline, including a sample file, is available alongside the used reference databases (Suppl. material 6) and the ASV sequence data (Suppl. material 7).

For the comparison of the efficacy of the ITS1/2 makers, we assessed the number of detected taxa per sample after rarefaction, but excluded any subsequent filtering steps to minimize pipeline bias (Suppl. material 5). This was only applied to 2019 samples where both, ITS1/2, were successfully amplified. The Jaccard similarity was calculated for each of the taxonomic levels (family, genus, species) to compare the taxon lists recovered by the ITS1/2 and *trnL*-P6 loop. We ultimately decided to limit our analysis to presence / absence data because there currently is no consensus whether *trnL*-P6 read numbers show a significant correlation with pollen counts in the eDNA sample (Deagle et al. 2019; Baksay et al. 2020; Polling et al. 2021).

Results

Bumblebee specimens and sequencing success

Of the 99 sampled bumblebees from 2019 (99 body swap samples + 18 corbicula pollen samples), 91 produced molecular data for all three plant barcoding markers. This dataset was used for foraging preference analysis and included the following bumblebee species, identified visually and via DNA barcoding: 40 *B. terrestris*, 24 *B. lapidarius*, 23 *B. pascuorum*, 3 *B. pratorum* and 1 *B. cryptarum* (Appendices 1, 2). Of the 81 corbicula pollen samples sampled from museum specimens (1968/69), we successfully generated NGS data of the *trnL*-P6 loop from 65 samples (39 *B. pascuorum*, 10 *B. veteranus*, 7 *B. distinguendus*, 5 *B. hortorum*, 3 *B. lucorum*, 1 *B. muscorum*).

Marker choice affects plant taxa detection

To assess the technical performance of ITS1/2 and the trnL-P6 loop, we minimized the filter steps to increase sensitivity (Tables 1, 2, Appendix 4). As a result, we detected 37 plant families, 96 genera, and 61 species in the 99 bumblebee pollen samples from 2019 (Tables 1, 2, Appendix 4). The barcoding markers identified distinct sets of plant taxa. Differences in taxonomic resolution between the ITS1/2 markers were mainly observed at the genus and species levels (Appendix 4). Although the Jaccard similarity at the genus level was 0.84 (Table 2), the ITS1/2 markers can be considered roughly equivalent, given that most variation at the genus level involved plant taxa found in only one specimen respectively, such as *Papaver* or *Clematis* (Appendix 4). However, it is worth mentioning that the ITS1 marker detected 15 plant genera not detected by ITS2, while ITS2 only identified three genera not detected by ITS1 (Appendix 5).

In addition to variation in the identified taxa, the ITS1/2 markers also exhibited discrepancies in the sum of presence detections of a taxon across all samples (Table 2). Aggregating the presence detections for all plant taxa revealed that the ITS1 marker outperformed the ITS2 marker by approximately 20% in terms of identifications at the genus and species levels (714 vs. 562) (Table 1). This trend persisted even when excluding the taxa exclusively detected by ITS1 from the count.

The *trnL*-P6 marker detected the most plant families (n=35), compared to ITS1 (n=32) and ITS2 (n=31), but fewer plant taxa at genus level (*trnL*-P6 n=42, ITS1 n=77, ITS2 n=65) and species levels (*trnL*-P6 n=13, ITS1 n=52, ITS2 n=45) (Table 1). Considering the detection counts, the *trnL*-P6 loop detected more individual family signals (n=637) as the ITS1/2 markers (454 and 436, respectively) across all samples (Table 1). However, ITS1/2 outperformed the *trnL*-P6 on the individual genus and species level detections (Table 1). The families Asteraceae, Malvaceae and Rosaceae were most affected by the low *trnL*-P6 resolution (Appendices 4, 5). The low Jaccard similarity of detections between the *trnL*-P6 loop and ITS1 or ITS2 on genus and species level can be primarily attributed to the low resolution of the *trnL*-P6 loop (Table 1, 2). In Asteraceae, for example, the resolution of the *trnL*-P6 loop is restricted to family level (except *Achillea*), while the ITS1/2 markers successfully distinguished between 23 genera (Appendix 4).

We also calculated the similarity between the ITS1 and ITS2 data of the same sample individually (1 vs. 1) instead of comparing the whole sample

Table 1. Aggregated plant taxa detection counts by pollen metabarcoding of 2019 samples (Cuxhaven, Germany). The detected plant taxa are aggregated from all samples of 2019 (n=99). The taxa presence detection count is calculated by summing up the number of detected plant taxa (frequency) per sample across all samples. Data originates from dataset without final two filtering steps to maximize taxa detection (Suppl. material 5).

	Plant taxa				Taxon presence detection sum		
	ITS1	ITS2	*trnL*-P6	combined	ITS1	ITS2	*trnL*-P6
Family	32	31	35	37	454	436	637
Genus	77	65	42	96	714	562	473
Species	52	45	13	61	523	407	196

Table 2. Jaccard similarity index of three metabarcoding markers of 2019 samples (Cuxhaven, Germany). The Jaccard similarity has been calculated based on a pooling of all samples of 2019 (n=99). Data originates from dataset without final two filtering steps to maximize taxa detection (Suppl. material 5).

	ITS1 vs. ITS2	ITS1 vs. *trnL*-P6	ITS2 vs. *trnL*-P6
Family	0.91	0.87	0.87
Genus	0.84	0.43	0.45
Species	0.71	0.28	0.34

pool (all ITS1 vs. all ITS2). Excluding genera exclusively being detected in either ITS1 or ITS2, the Jaccard similarity of bumblebee samples from 2019 (male and female, body and corbicula) between ITS1 and ITS2, per specimen, is 0.59 (n=110) (Suppl. material 7). The similarity between the markers rises asymptotically to 0.81 at a cutoff rate of 400 reads and reaches saturation at a similarity index of 0.88 at a cutoff rate of 870 reads (Suppl. material 7).

Pollen metabarcoding as a tool to reveal plant-pollinator interactions

Data used to analyze the plant-pollinator interactions was filtered more strictly to exclude rare and infrequent plant signals that may be of minor importance in terms of their impact on the bumblebee colonies nutritional status (Table 3; Fig. 1, Appendices 1–3). The plant taxa identification in ITS1 (n=42) and ITS2 (n=45) were at least on the genus level (Appendices 1, 2). In *trnL*-P6, the highest taxonomic rank of the identified plant taxa (n=47) was either on family (n=12) or genus level (n=35) (Appendix 3). In detail, successful detections at genus or family level in the *trnL*-P6 loop, which were not detected by ITS1 and ITS2, can be partly attributed to *Atriplex*, *Alnus*, *Robinia*, *Rumex*, *Acer*, *Pinus* or Salicaceae (Appendix 4). In body samples (n=2) of *B. pratorum* the additionally detected taxa by the *trnL*-P6 loop in comparison to ITS1 and ITS2 were Convolvulaceae, Cucurbitaceae, Rosaceae, Ericaceae: *Calluna*, Fabaceae: *Lotus*, Lamiaceae: *Mentha* and Onagraceae: *Oenothera* (Appendix 3).

A quantitative count of the pollination network links (=connection) reveals that the markers show a different trend (Table 3). The median of detected taxa (statistical median of number of network links across specimens of the same species) in the *trnL*-P6 marker is always higher or equal to the other markers (Table 3). In contrast to most of the ITS1 or ITS2 evaluations (except *B. pascuorum* in ITS2), *trnL*-P6 recovered more taxa in the corbicula samples than the body samples (Table 3). Despite the high abundance of some taxa, e.g.

Scorzoneroides, *Tanacetum*, *Lotus* and *Lythrum*, detected in more than 10 specimens in at least one bumblebee species by ITS1/2 (Appendices 1–3), the median number of plant taxa on individual female worker bumblebees was 2.5 to 4 for ITS1 and ITS2 (where n > 3 bumblebees, Table 3). The old corbicula samples (1968/69) did not recover less taxa when compared to the 2019 samples (where n > 3 bumblebees, Table 3). Plant taxa were found in a median of two to three bumblebee body samples (where n > 3 bumblebees, Table 3).

Table 3. Plant taxa counts in bumblebee samples. The number of bumblebee connections describes the median number of plant taxa found in one sample (number of pollination network links from bumblebee to plant taxa). The number of median plant connections describes the number of samples each respective plant taxa was found in (number of pollination network links from plant taxa to bumblebees). Samples from 1968/69 (=old) have been separated from the samples of 2019. The taxonomic identification level was always to genus in the ITS1 and ITS2 marker and to genus or family level in the *trnL*-P6 marker. The interquartile range (values in brackets) is only given if the number of samples (n) was greater than 2.

pollen source	bumblebee species (females only)	median bumblebee connections						median plant connections					
		ITS1		ITS2		*trnL*–P6		ITS1		ITS2		*trnL*–P6	
body	*B. terrestris* (n=27)	3	(2–5)	3	(2–4)	4	(2–6)	3	(1–4)	2	(1–3.25)	2.5	(1–7.5)
	B. lapidarius (n=24)	4	(2.75–5)	2.5	(1.75–4.25)	4	(2.75–5)	2.5	(1–5)	3	(2–6)	3	(2–9)
	B. pascuorum (n=23)	4	(3–5.5)	3	(2–4)	4	(3.5–5)	2	(2–4)	3	(1–3.5)	2.5	(1–5.75)
	B. pratorum (n=2)	1.5	–	1	–	7	–	1.5	–	2	–	2	–
	B. cryptarum (n=1)	1	–	3	–	7	–	1	–	1	–	1	–
corbicula	*B. terrestris* (n=10)	2	(2–3.75)	2	(2–3.75)	4.5	(3.25–5.75)	1	(1–2)	2	(1–3)	2.5	(1–4)
	B. lapidarius (n=4)	1.5	(1–2.75)	1.5	(1–3)	7	(5–7.5)	1	(1–1.75)	1	(1–1.25)	2	(1–2.25)
	B. pascuorum (n=4)	2	(2–2.5)	3.5	(3–4)	6	(5–7.5)	1	(1–2)	1.5	(1–2)	1	(1–2)
	B. pascuorum (old) (n=39)	–	–	–	–	5	(3.5–8)	–	–	–	–	2	(1–9)
	B. veteranus (old) (n=10)	–	–	–	–	5	(4–7.5)	–	–	–	–	3	(1–4)
	B. distinguendus (old) (n=7)	–	–	–	–	5	(3–7)	–	–	–	–	1.5	(1–4)
	B. hortorum (old) (n=5)	–	–	–	–	7	(4–10)	–	–	–	–	2	(1–2.5)
	B. lucorum (old) (n=3)	–	–	–	–	13	(10.5–13)	–	–	–	–	1	(1–3)
	B. muscorum (old) (n=1)	–	–	–	–	2	–	–	–	–	–	1	–

Comparison of corbicula and body pollen samples

85% (ITS1) and 75% (ITS2) of the plant taxa, at genus level, found in the corbicula samples (n=18) from female bumblebees caught in 2019 (*B. terrestris*, *B. lapidarius* and *B. pascuorum*) can also be detected in the respective body samples of the same specimen (Suppl. material 7). On family level the overlap rises to 91% for ITS1 and 78% for ITS2 (Suppl. material 7). Due to high similarity indices between corbicula and body samples of the same individual in ITS1/2, the corbicula samples can be broadly viewed as a subset of the body samples. For the *trnL*-P6 loop, where a higher number of plant taxa has been detected in the corbicula samples, 75% of the plant taxa detected in body samples have been found in the corbicula samples of the same specimen (Suppl. material 7). At family level, the overlap increases to 79% (Suppl. material 7).

Comparison between historic and recent bumblebee samples

In general, the most often visited taxa in 2019 were also detected in the 1968/69 samples and vice versa, albeit at a different frequency (Fig. 1). In detail, each of the well-covered plant families (s > 20) was found on each of the well-represented bumblebee species (n > 20) included in this study (Fig. 1). However, trends

of plant-pollinator interactions in the 2019 samples versus the samples from 1968/69 using the *trnL*-P6 loop plant barcode marker are different. In detail, the ratio of recent / historic interactions per plant family (s > 20) varies with Asteraceae, Ericaceae and Lythraceae showing, relatively, more recent interactions and Fabaceae and Oleaceae showing the smallest ratio of recent interactions (Fig. 1). *Phaseolus* and *Lathyrus* (both Fabaceae), with one exception, can only be found on 1968/69 bumblebees. The difference in visitation pattern for *B. pascuorum* is especially striking, as we could rarely find *Vicia* and *Trifolium* plant-pollinator interactions in recent data, compared to historic *B. pascuorum* specimens (Fig. 1). *Bombus pascuorum* visited the same plant taxa (with at least 3 interactions in historic and recent data) in historical and recent collections, except for *Phaseolus*, which was additionally detected in the 2019 survey (Appendix 3).

Discussion

Biodiversity loss, particularly the decline of pollinators and its impact on ecosystems, is a pressing contemporary issue. Understanding the potential causes behind this decline is highly relevant. In the following, we discuss changes in flower-visiting behavior over a span of approximately 50 years using museomics and metabarcoding of pollen. Our findings reveal a decline in interactions with Fabaceae, which may contribute to the decline of numerous rare species. Additionally, the effectiveness and consistency of our method across various barcoding markers are demonstrated. Subsequently, a detailed discussion of the results is provided.

Comparison of recent and historic *Bombus* samples

To the best of our knowledge, this is the first pollen metabarcoding study of historic bumblebee pollen samples dating back ~50 years and the only bumblebee metabarcoding study reporting results from multiple endangered *Bombus* species. Existing pollen metabarcoding studies are primarily focused on *B. terrestris* (Wilkinson et al. 2017; Biella et al. 2019; Potter et al. 2019; Bänsch et al. 2020; Piko et al. 2021; Bontšutšnaja et al. 2021). This can possibly be explained by the difficulties associated in locating rare bumblebee species in the field. In the study of Beyer et al. (2020), only 1.2% of the bumblebees caught belonged to an endangered bumblebee species (Westrich et al. 2011). This underlines the usefulness of historic natural history collections in reconstructing trophic interactions between species, such as pollinator-plant interactions, especially when rare species are included (Scheper et al. 2014). However, it is important to recognize that plant-pollinator interactions must be supplemented with knowledge about the effects of shifts in flowering time, climate change, parasites or diseases, pesticides, and competition before conservation measures can be taken. (Goulson et al. 2015; Miller-Struttmann et al. 2015; Marshall et al. 2018; Soroye et al. 2020).

Our data on shifts in flower visitations revealed trends that differ between current and historical bumblebee specimens. The bumblebee species caught in 2019 are commonly reported to be present in urban environments and display a highly generalist foraging behavior (Banaszak-Cibicka and Żmihorski 2012; Zajdel et al. 2019; Sikora et al. 2020). This is different from two species only found in the historic samples (*B. distinguendus* and *B. muscorum*), which have been reported to be amongst the bumblebees with the narrowest dietary breadth

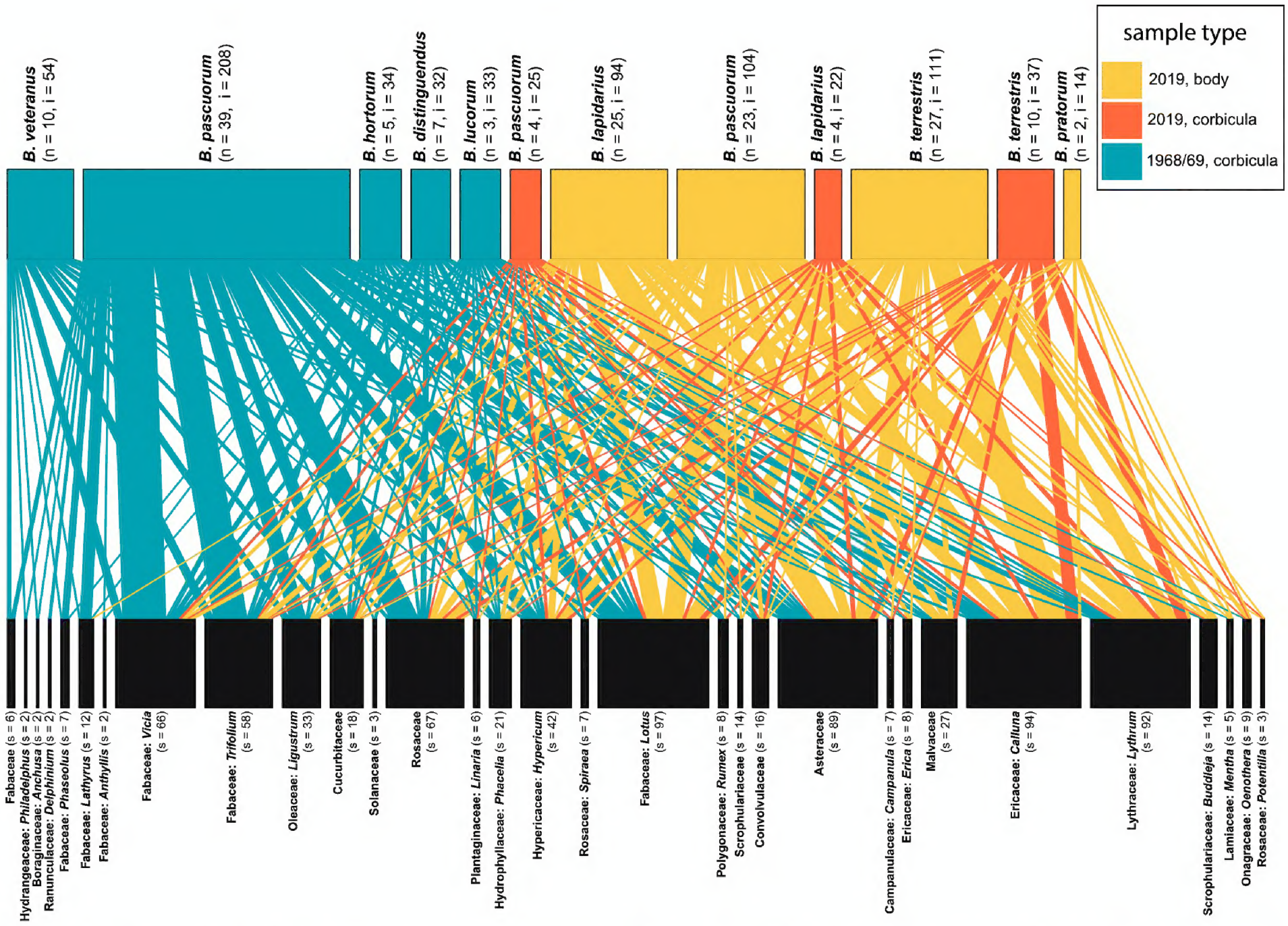
sample type
2019, body
2019, corbicula
1968/69, corbicula
B. veteranus (n = 10, i = 54)
B. pascuorum (n = 39, i = 208)
B. hortorum (n = 5, i = 34)
B. distinguendus (n = 7, i = 32)
B. lucorum (n = 3, i = 33)
B. pascuorum (n = 4, i = 25)
B. lapidarius (n = 25, i = 94)
B. pascuorum (n = 23, i = 104)
B. lapidarius (n = 4, i = 22)
B. terrestris (n = 27, i = 111)
B. terrestris (n = 10, i = 37)
B. pratorum (n = 2, i = 14)
Fabaceae (s = 6)
Hydrangeaceae: Philadelphus (s = 2)
Boraginaceae: Anchusa (s = 2)
Ranunculaceae: Delphinium (s = 2)
Fabaceae: Phaseolus (s = 7)
Fabaceae: Lathyrus (s = 12)
Fabaceae: Anthyllis (s = 2)
Fabaceae: Vicia (s = 66)
Fabaceae: Trifolium (s = 58)
Oleaceae: Ligustrum (s = 33)
Cucurbitaceae (s = 18)
Solanaceae (s = 3)
Rosaceae (s = 67)
Plantaginaceae: Linaria (s = 6)
Hydrophyllaceae: Phacelia (s = 21)
Hypericaceae: Hypericum (s = 42)
Rosaceae: Spiraea (s = 7)
Fabaceae: Lotus (s = 97)
Polygonaceae: Rumex (s = 8)
Scrophulariaceae (s = 14)
Convolvulaceae (s = 16)
Asteraceae (s = 89)
Campanulaceae: Campanula (s = 7)
Ericaceae: Erica (s = 8)
Malvaceae (s = 27)
Ericaceae: Calluna (s = 94)
Lythraceae: Lythrum (s = 92)
Scrophulariaceae: Buddleja (s = 14)
Lamiaceae: Mentha (s = 5)
Onagraceae: Oenothera (s = 9)
Rosaceae: Potentilla (s = 3)

Figure 1. Plant-pollinator inference network of historic and recent bumblebee specimen by *trnL*-P6 pollen metabarcoding. Corbicula pollen was sampled from bumblebees caught in 1968/69 (blue) and bumblebees caught in 2019 (orange). In addition, body pollen was sampled from all bumblebees caught in 2019 (yellow). The width of the colored bars reflects the sum of unique interactions (i) with plant taxa for all specimen (n) of the respective sample type (color). The width of the black bars reflects the total number of bumblebee specimens (s) in which the respective plant taxa has been found. Plant taxa were ordered to minimize network overlap. To avoid clutter, plant taxa found in less than two samples and bumblebee species represented by less than two specimens were omitted. Taxa represented by family names showed insufficient resolution in the *trnL*-P6 maker (e.g., Asteraceae).

(Wood et al. 2021). On the other hand, *B. lapidarius*, *B. terrestris* and *B. pascuorum*, in combination, have been reported in other areas with high population density, indicating their status as hemerophiles (Vray et al. 2019). Although we could show their generalist foraging strategy, most plant taxa could only been found on a small subset of all analyzed samples (Table 3). This indicates that a higher sampling depth is required to fully characterize the foraging habits. We can hypothesize that the greater frequency of *Calluna* flower visitation is due to the establishment of a strictly protected heathland that was not yet a formal protected area in 1969 (Wagner 1969). The higher visitation frequency of *Lythrum* could be a result of an increased amount of drainage channels, which provide an ideal habitat, due to intensified agriculture (Wagner 1969; Dierssen 1997). While difficult to predict, a shift in flowering time could also have played a role in floral availability (Rafferty et al. 2020), which, however, was not tested here.

Our analysis of recent and historic plant-pollinator interactions revealed distinct floral visits (Fig. 1). While the overlap of analyzed species is restricted to *B. pascuorum*, it can be inferred that Fabaceae are an important part of bumblebee diet

(Wood et al. 2021), particularly for *B. lapidarius* (Hülsmann et al. 2015). Therefore, if available, all accessible floral resources from the Fabaceae family would have been used by *B. pascuorum* and *B. lapidarius*, as observed in *Lotus* (Fig. 1). In contrast to our results, bumblebee diversity was more extensive in the Cuxhaven area in 1968/69 (Wagner 1969). An important consideration for our results is whether the species is truly absent from the area, or we just could not find it. Therefore, we will discuss probable causes for its absence in our 2019 sampling effort.

B. ombus lucorum is reported to be a generalist forager (Wood et al. 2021), which aligns with our data (Fig. 1, Appendix 3). Reports of *B. lucorum* in the area are inconsistent. It has been classified as an abundant species in the area (Witt 2016), however, other studies report relatively low catch rates of 1.6% (Persson et al. 2015). In 1968/69 it accounted for 4.5% of the bumblebees captured (Wagner 1969). Overall, we conclude that *B. lucorum* was likely missed in 2019 due to insufficient sampling. Another possible explanation for the absence of *B. lucorum* in our samples is that the number of hot days (>25 °C) was higher than in 1968/69 (DWD 2022) and shortened the colony's lifecycle (Maebe et al. 2021).

Our data confirms that *B. distinguendus* has a preference for Fabaceae (Diekötter et al. 2006; Witt 2016). However, we also observed the use of alternative pollen sources (Vray et al. 2017; Phelan et al. 2021), such as *Rosa* and *Calluna* (Appendix 3). In contrast to reports between 1909 and 1969 (Alfken 1913; Wagner 1969; Rasmont et al. 2015), *B. distinguendus* is assumed to be absent from most parts of Lower Saxony nowadays (Sprichardt 2010; Rasmont et al. 2015; Witt 2016), which may explain the absence in our study (Fig. 1). A similar trend of habitat destruction or extinction was also found in multiple other countries (Goulson et al. 2005; Charman et al. 2010; Dupont et al. 2011; Bommarco et al. 2012; Rasmont et al. 2015; Rollin et al. 2020). Common recommendations to protect *B. distinguendus* include modifying mowing practices of open grassland areas and converting pastures into species-rich grassland, provided suitable nesting sites are available (Charman et al. 2010; Witt 2016; Phelan et al. 2021).

The decline of *B. hortorum*, which is generally not considered a rare species in Germany, between 1959–1962 and 1968–1969 in the Cuxhaven area, can be attributed to land use changes (Wagner 1969). Although it was present in 2007 and 2009 in the Cuxhaven area, it was only observed in 2 out of 85 sampling days (Sprichardt 2010). We were unable to capture any *B. hortorum* individuals in 2019. *Bombus hortorum* is known to show an extreme dietary preference for *Trifolium* (Goulson et al. 2008; Kleijn and Raemakers 2008), and likely also other Fabaceae species. Interactions between bumblebees and *Trifolium* were underrepresented in our 2019 data, compared to the data from 1968–1969 (Fig. 1). This possibly hints at the fact that the land use changes were accompanied by a change in floral availability. While some studies have not demonstrated a dietary focus on Fabaceae (Wood et al. 2021), it is challenging to compare results across dietary studies as many parameters are not being controlled for (i.e., floral availability).

It has been hypothesized that *B. veteranus* is closely associated with Fabaceae (Goulson et al. 2008; Wood et al. 2021), and our data confirms this finding (Appendix 3). However, another study showed *B. veteranus* to be tightly associated with Cardueae (Vray et al. 2017), which was not reflected in our data (Fig. 1). Our data also revealed interactions with plants associated with anthropogenic influence, such as *Ligustrum* (Appendix 3). This suggests that the habitat of *B. veteranus* may be threatened by nearby land use changes, espe-

cially urbanization. *Bombus veteranus* is considered a rare species in Germany (Westrich et al. 2011) and has experienced rapid decline in Belgium (Rollin et al. 2020). In the Cuxhaven area, its last record is from 1969 (Wagner 1969). In Lower Saxony only one large population is known (Witt 2016).

In summary, the detected floral interactions of bumblebees caught in 2019 have shifted away from many Fabaceae genera. This is important for three reasons: 1) Host plant availability has been identified as the main driving factor of wild bee decline (n = 57 species), with Fabaceae dependent species (29 out of 57) showing the highest decline (Scheper et al. 2014). 2) Herbaceous Fabaceae, such as *Vicia* and *Trifolium*, are of principal importance for many rare bumblebees (Bäckman and Tiainen 2002; Goulson et al. 2005; Goulson et al. 2008; Kleijn and Raemakers 2008; Dupont et al. 2011; Timberlake et al. 2019; Wood et al. 2019; Sikora et al. 2020; Wood et al. 2021). And finally, 3) Fabaceae has been found to be the most effective plant family in mitigating negative effects of urbanization, promoting their usefulness also in urban landscapes (Hülsmann et al. 2015).

Plant barcoding markers in pollen metabarcoding

We tested three genetic markers, including the trnL-P6 loop, positioned between the *trnL* (UAA) exon 1 and the *trnL* (UAA) exon 2 (Taberlet et al. 2007), which has been utilized in multiple pollen and honey metabarcoding studies (Chiara et al. 2021; Milla et al. 2021; Polling et al. 2021). Compared to studies using pollen microscopy, it shows worse taxonomic resolution in the Asteraceae family and approximately the same taxonomic resolution in other plant families (Wood et al. 2019; Wood et al. 2021).

Comparing the trnL-P6 loop with ITS1/2 revealed two important findings. First, in accordance with Milla et al. (2021), we observed that the *trnL*-P6 loop is able to capture greater diversity at the family level than the ITS1/2 marker (Table 1). This increased diversity can be attributed to the trnL-P6 loop's ability to amplify degraded DNA due to its shorter length (Valentini et al. 2009). There are multiple possible explanations for the additionally detected taxa in *trnL*-P6 compared to ITS1/2 in samples of 2019. Plant material attached could be accidentally deposited in the corbicula during combing alongside with pollen. Regurgitated nectar, used to fixate pollen on the corbicula (Thorp 2000), which also contains ingested pollen (Owen et al. 2013), could also add traces of degraded DNA to the pollen package. This could also explain why, in contrast to the ITS1/2 marker, the trnL-P6 loop was able to identify more plant taxa in the corbicula samples, compared to the body samples (Table 3).

Second, supported by Polling et al. (2021), in contrast to Milla et al. (2021), our results show that the ITS1/2 markers recover a higher number of taxa at species and genus rank (Table 1). After the manual curation of SINTAX results, the *trnL*-P6 loop identified only ~60% of the genera found by ITS1/2 in recent samples (Table 1).

These results demonstrate that sequencing shorter DNA fragments, such as the *trnL*-P6 loop, alongside with longer DNA fragments, such as the ITS1/2 barcode marker, will yield different insights and are well worth exploring. Unfortunately, we could not find comparable studies in literature and further controlled experiments are required to understand the differential detections of ITS1/2 and trnL-P6. In summary, the *trnL*-P6 loop was generally able to recover a higher taxonomic breadth, while the ITS1/2 maker was generally able to recover a higher taxonomic depth.

Demonstrating the utility of ITS1 in metabarcoding

Our study shows that the ITS1 possesses favorable attributes, compared to the ITS2 marker. This can be demonstrated by more detected taxa on species and genus level, as well as the overall higher number of taxa in all samples (Table 1). The higher taxonomic resolution aligns with previous results (Wang et al. 2015; Kolter and Gemeinholzer 2021b). The increased richness of taxa per sample suggests a more even amplification profile of mixed samples, potentially explained by the more conserved flanking regions of ITS1 (Wang et al. 2015; Kolter and Gemeinholzer 2021a). Another possible explanation could be the, on average, lower GC content of ITS1 compared to ITS2 (Wang et al. 2015), resulting in less stable secondary template structures during PCR. Our study, due to improvements in methodology (Cheng et al. 2016; Kolter and Gemeinholzer 2021a), contrasts the findings of Chen et al. (2010), who excluded ITS1 as a barcode candidate due to amplification problems. The overall performance of ITS1 also contradicts the study of Gous et al. (2019), which, however, used primers which were also designed for fungal amplification (White et al. 1990; Kolter and Gemeinholzer 2021a). Subsequently, their findings are possibly a result of preferential amplification of fungal DNA and must be verified with plant-specific primers.

One disadvantage of ITS1 is the presence of extremely long ITS1 sequences in certain Gymnosperms (Cheng et al. 2016), which currently exceeds the technical limits of the Illumina sequencing platform (2×300 bp). ITS1 has been used sparingly in pollen metabarcoding studies (Pornon et al. 2016; Gous et al. 2019; Baksay et al. 2020; Gous et al. 2021), and further investigations, ideally including mock communities, are required, before it can be recommended over ITS2. In this context, it is important to mention that the number and quality of taxa recovered from any eDNA sample by metabarcoding depend heavily on the metabarcoding pipeline used (Pauvert et al. 2019). Finally, we conclude that the application of ITS1 in pollen metabarcoding studies needs more comparative studies but shows promise.

Conclusion

In conclusion, our study was able to show differences in foraging trends of bumblebees caught in 1968/69 and 2019, contributing to our understanding of their interaction with foraging resources, despite their current absence from the study area.

Moreover, our findings demonstrate that the *trnL*-P6 loop had poorer taxonomic resolution compared to the ITS1/2 marker, but could detect more plant-pollinator interactions. We furthermore showed that the ITS1 marker performs at least comparably to the ITS2 marker and holds promise for effective application in plant metabarcoding studies.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Andreas Kolter: Writing - original draft; Writing - review and editing; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. Martin Husemann: ; Writing - original draft; Writing - review and editing; Investigation; Project administration; Resources. Lars Podsiadlowski: Writing - review and editing; Investigation; Resources. Birgit Gemeinholzer: Conceptualization; Writing - review and editing; Writing - original draft; Funding acquisition; Project administration; Resources; Supervision.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information. Raw sequence reads are deposited in NCBI BioProject PRJNA841517.

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Appendix 1

Table A1. ITS1 plant taxa detections in samples from 2019.

Family	Genus	Species	*B. terrestris* (f), n=27	*B. terrestris* (m), n=13	*B. terrestris* (f)LP, n=10	*B. lapidarius* (f), n=24	*B. lapidarius* (f)LP, n=4	*B. pascuorum* (f), n=23	*B. pascuorum* (f)LP, n=4	*B. pratorum* (f), n=2	*B. pratorum* (m), n=1	*B. cryptarum* (f), n=1
Amaryllidaceae	*Allium*	*ampeloprasum*		1								
	Halimione	*portulacoides*		1		2						
Asteraceae	*Achillea*	*millefolium*	2	2	1	3						
	Artemisia		3	4	1	4		2				
	Bidens		1									
	Centaurea	*cyanus*	1			2	1					
	Cirsium	*vulgare*	4	3	1							
	Crepis	*capillaris*	1	1	1	1		2				
	Dahlia		1	1		1		3				
	Eupatorium	*cannabinum*	1	2				2				
	Helianthus	*annuus*	1	1				1				
	Hypochaeris	*radicata*	1	2	2	1		2				
	Leontodon			1		5						
	Liatris		4	2	1	3		4				
	Scorzoneroides	*autumnalis*	4	1	1	18		2				
	Senecio	*inaequidens*	1	2				2		1		
	Tanacetum	*vulgare*	7	7	1	15		5			1	
Balsaminaceae	*Impatiens*	*glandulifera*						1				
Boraginaceae	*Borago*	*officinalis*	1			1						
Campanulaceae	*Campanula*			2	1							
Convolvulaceae	*Calystegia*	*sepium*		2								
Ericaceae	*Calluna*	*vulgaris*	2	4	7	7	3	6	2			
	Erica	*tetralix*	4	2	2	5	1	5	1			
Fabaceae	*Lotus*		6	1		11	2	16	1			
		pedunculatus	5	1		9	2	18	1			
	Trifolium	*arvense*	1	1		1		1				
		repens	2				1	1				
Hydrangeaceae	*Hydrangea*		3		1			2				
Hydrophyllaceae	*Phacelia*	*tanacetifolia*	2	1	1	1		4				
Hypericaceae	*Hypericum*		5		4			4	2			
Lythraceae	*Lythrum*	*salicaria*	1	9	1	4		18	2	2	1	1
Malvaceae	*Alcea*			1				2				
		rosea	4	2		1		4				
	Malva			2								
Oleaceae	*Ligustrum*		1	2								
Onagraceae	*Oenothera*	*biennis*	3	2	2	1		1				
Plantaginaceae	*Linaria*							1	1			
Plumbaginaceae	*Limonium*	*vulgare*		1		1						
Rosaceae	*Potentilla*	*anserina*	1			1	1					
	Rosa		3		3			1				
Rosaceae	*Rubus*		1	1				2				
Solanaceae	*Solanum*	*dulcamara*						2	1			

Taxa are not listed in a hierarchical manner (e.g., *Lotus* and *Lotus pedunculatus* counts originate from different ASVs). Each positive count represents a presence signal which persisted after all filtering steps. Bumblebee species are split by sex (f/m) and sample location on the bumblebee's body (body, LP = corbicula).

Appendix 2

Table A2. ITS2 plant taxa detections in samples from 2019.

Family	Genus	Species	*B. terrestris* (f), n=27	*B. terrestris* (m), n=13	*B. terrestris* (f)LP, n=10	*B. lapidarius* (f), n=25	*B. lapidarius* (f)LP, n=4	*B. pascuorum* (f), n=23	*B. pascuorum* (f)LP, n=4	*B. pratorum* (f), n=2	*B. pratorum* (m), n=1	*B. cryptarum* (f), n=1
Apiaceae	*Pimpinella*		1									
Asteraceae	*Achillea*		2	2	1			2				
	Artemisia	*vulgaris*	2	3								
	Bidens		1									
	Centaurea	*cyanus*	1			2	1					
	Cirsium	*vulgare*	3	3								
	Crepis	*capillaris*	1		1	1						
	Dahlia		1	1				1				
	Eupatorium	*cannabinum*	1	1				2				
	Hypochaeris			1	2	6						
	Leontodon					3						
	Liatris		4	2	2	2		4				
	Scorzoneroides	*autumnalis*	2		1	14		1				
	Senecio		2	3				1				
	Tanacetum		6	5	4	17	1	4	2			1
Balsaminaceae	*Impatiens*	*glandulifera*						1				
Boraginaceae	*Borago*	*officinalis*	1									
Brassicaceae	*Raphanus*	*sativus*	2	1		2						
Ericaceae	*Calluna*	*vulgaris*	17	3	6	6	2	4	2			1
	Erica	*tetralix*	1	1	1			3	1			
Fabaceae	*Lotus*		5	2	1	1	2	16	2		1	
		corniculatus	2	1	1	8	2	1	1			
		pedunculatus	4	1	1	7	2	16	2			
	Ononis	*spinosa*	1									
	Trifolium							1				
		repens	1				1	1				
Hydrangeaceae	*Hydrangea*	*paniculata*	1	1				1				
		serrata	3		1			2				
Hydrophyllaceae	*Phacelia*	*tanacetifolia*	2		1							
Hypericaceae	*Hypericum*		4		3							
Lamiaceae	*Mentha*		3	1								
Lythraceae	*Lythrum*	*salicaria*	11	1	4	6	1	19	4	2	1	1
Malvaceae	*Alcea*		2	1		1		3				
	Malva	*moschata*		2								
Oleaceae	*Ligustrum*	*ovalifolium*	1	1								
Onagraceae	*Oenothera*		2	1	3			1				
Plantaginaceae	*Linaria*	*vulgaris*						1	1			
Plumbaginaceae	*Limonium*	*vulgare*		1								
Rosaceae	*Potentilla*	*anserina*	1			1	1					
	Rosa		5		3		1	2				
	Rubus			1								
Scrophulariaceae	*Buddleja*	*Davidii*	4	1				3	1			
Solanaceae	*Solanum*	*Dulcamara*	3					3	1			

Taxa are not listed in a hierarchical manner (e.g., *Lotus* and *Lotus pedunculatus* counts originate from different ASVs). Each positive count represents a presence signal which persisted after all filtering steps. Bumblebee species are split by sex (f/m) and sample location on the bumblebee's body (body, LP = corbicula).

Appendix 3

Table A3. *trnL*-P6 plant taxa detections in samples from 1968 – 2019.

Family	Genus	Species	*B. pascuorum* (f), n=23	*B. pascuorum* (f) LP, n=4	*B. pascuorum* (f) (old), n=39	*B. terrestris* (f), n=27	*B. terrestris* (m), n=13	*B. terrestris* (f) LP, n=10	*B. lapidarius* (f), n=25	*B. lapidarius* (f) LP, n=4	*B. veteranus* (f) (old), n=10	*B. distinguendus* (f)(old), n=7	*B. hortorum* (f) (old), n=5	*B. pratorum* (f), n=2	*B. pratorum* (m), n=1	*B. lucorum* (f) (old), n=3	*B. muscorum* (f) (old), n=1	*B. cryptarum* (f), n=1
Asparagaceae	*Asparagus*				1													
Poaceae			1	1	3		1			1						1		
Anacardiaceae	*Cotinus / Rhus*				2							1						
Apiaceae					1													
Asteraceae			9	1	16	12	7	4	23	3	4	3	3	2		2		
Betulaceae	*Alnus*								1			1						
Boraginaceae	*Anchusa*				1						1							
Brassicaceae	*Cardamine*				1													
Campanulaceae	*Campanula*			2	1	1	2	1										
Convolvulaceae			3	1	3	1	3			2		1		1		1		
Cucurbitaceae			2	1	1			1	3		4	2	2	1		1		
Ericaceae	*Calluna*	*vulgaris*	16	3	9	23	9	1	14	3	4	4	3	2		3		1
	Erica	*tetralix*	3	1	1	1	1									1		
Fabaceae					3						3							
	Anthyllis	*vulneraria*			1							1						
	Lathyrus	*pratensis*	1		9						1		1					
	Lotus		16	3	21	12	5	5	15	2	4	5	4	2	1	2		1
	Phaseolus	*vulgaris*			5						1		1					
	Robinia	*pseudoacacia*				1							1					
	Styphnolobium	*japonicum*										1						
	Trifolium		6	2	27	1	2	1	3	1	7	4	3			1	1	1
	Vicia		2	2	35	5	1		3	1	6	4	4			3	1	1
Hydrangeaceae	*Philadelphus*				1						1							
Hydrophyllaceae	*Phacelia*	*tanacetifolia*	4		5	1	2	1	4		1	1	1			1		
Hypericaceae	*Hypericum*		1	2	12	8	1	3	5	2	3	2	1			2		
Lamiaceae					1													
	Mentha		1			2							1	1				
Lythraceae	*Lythrum*	*salicaria*	21	3	8	14	9	9	12	4	3	1	2	2	1	3		1
Malvaceae			5		3	4	5	3	1		2		1		1	2		
Oleaceae	*Ligustrum*		2		13	4	2	1	2		6		2			1		
Onagraceae	*Oenothera*					3	2	1		1				1		1		
Plantaginaceae	*Linaria*	*vulgaris*	1	1	1		1						1			1		
Plumbaginaceae	*Limonium*						1		1									
Polygonaceae	*Fagopyrum*	*esculentum*			1													
Polygonaceae	*Rumex*		1		1			2					2			2		
Ranunculaceae	*Delphinium*				2													
Rosaceae			6	1	23	9	3	4	5	2	3	4	2	2	1	2		1
	Potentilla	*anserina*				1			1	1								
	Spiraea			1	2	2	1									1		1
Scrophulariaceae			1		1	1										1		
	Buddleja		3	1	2	6			2									
Solanaceae					1											2		
Pinaceae	*Pinus*		2		1	2	2		1			1	1		1			
Taxaceae	*Taxus*				1													

Taxa are not listed in a hierarchical manner (e.g., *Lotus* and *Lotus pedunculatus* counts originate from different ASVs). Each positive count represents a presence signal which persisted after all filtering steps. Bumblebee species are split by sex (f/m) and sample location on the bumblebee's body (body, LP = corbicula). Bumblebee samples from the 1968/69 period (=old) are always referring to corbicula samples.

Appendix 4

Table A4. Biodiversity exploration.

Family	Genus	Species	ITS1	ITS2	*trnL*-P6
Amaryllidaceae	*Allium*				1
		ampeloprasum	3	1	
Poaceae					5
	Lolium		3		
Amaranthaceae	*Atriplex*				5
	Chenopodium			1	4
		album	1		
	Halimione	*portulacoides*	3	6	
Araliaceae	*Hedera*	*helix*			3
Apiaceae					1
	Anethum	*graveolens*	1	2	
	Pastinaca	*sativa*	3	1	
	Pimpinella		1	2	
		saxifraga		1	
	Torilis	*japonica*	1		
Asteraceae					62
	Achillea		26	10	19
		millefolium	17		
	Artemisia		28		
		vulgaris		10	
	Bellis	*perennis*	1		
	Bidens		2	1	
	Centaurea	*cyanus*	8	4	
	Cirsium	*vulgare*	10	7	
	Crepis	*capillaris*	17	4	
	Dahlia		13	4	
	Eupatorium	*cannabinum*	9	6	
	Glebionis	*coronaria*	2		
	Helianthus	*annuus*	10	6	
	Hieracium			10	
		umbellatum	10		
	Hypochaeris	*radicata*	29	17	
	Jacobaea		2		
		maritima	1		
	Leontodon		11		
		saxatilis		3	
	Leucanthemum		3	2	
	Liatris		15	16	
	Scorzoneroides				
		autumnalis	39	27	
	Senecio			10	
		inaequidens	15		
	Solidago		2	2	
	Tagetes		1		
	Tanacetum		51	51	
		vulgare	45		
	Tripleurospermum	*maritimum*	1		
Balsaminaceae	*Impatiens*	*glandulifera*	1	1	2
Betulaceae	*Alnus*				4
	Carpinus	*betulus*		1	
Bignoniaceae	*Catalpa*		2		
		ovata		1	
	Borago				1
		officinalis	2	2	
	Echium	*plantagineum*	1		
	Symphytum	*officinale*	1		

Family	Genus	Species	ITS1	ITS2	*trnL*-P6
Brassicaceae					4
	Brassica	*rapa*	1		
	Raphanus	*sativus*	8	7	
Campanulaceae	*Campanula*		5		14
		rotundifolia		7	
	Lobelia				1
	Jasione	*montana*	1		
Convolvulaceae					9
	Calystegia	*sepium*	2	6	
Crassulaceae					6
	Sempervivum		1	1	
Ericaceae	*Calluna*	*vulgaris*	64	50	50
	Erica	*tetralix*	26	13	10
Fabaceae	*Hedysarum*				1
	Lathyrus	*pratensis*	1	1	7
	Lotus				53
		corniculatus	12	45	
		pedunculatus	55	51	
	Ononis	*spinosa*	1	2	5
	Robinia	*pseudoacacia*			2
	Trifolium		18	6	23
		arvense	9	1	
		pratense	1		
		repens	6	4	
	Vicia				4
Fagaceae			1		
	Fagus			1	2
Hydrangeaceae	*Hydrangea*		15	20	
		paniculata		7	
		quercifolia		2	
	Philadelphus				2
Hydrophyllaceae	*Phacelia*	*tanacetifolia*	11	6	34
Hypericaceae	*Hypericum*		18	8	17
Lamiaceae	*Clinopodium*				2
	Galeopsis				4
	Lycopus		1		
		europaeus		1	
	Mentha		6	7	7
	Physostegia				1
Lythraceae	*Lythrum*	*salicaria*	60	66	64
Malvaceae					37
	Alcea			8	
		rosea	14		
	Malva		3		
	Malva	*moschata*		3	
	Tilia		1		
Oleaceae	*Ligustrum*		9		27
		ovalifolium		8	
Onagraceae	*Chamaenerion*	*angustifolium*	2	2	
	Oenothera			10	10
	Oenothera	*biennis*	9		
Orobanchaceae	*Melampyrum*	*pratense*			1
	Odontites			3	
		vulgaris	3	2	
Papaveraceae	*Papaver*	*rhoeas*	1		
Plantaginaceae	*Digitalis*	*purpurea*	1	1	
	Linaria		4		
		vulgaris		2	9
	Plantago	*lanceolata*		3	
Plumbaginaceae	*Limonium*				9

Family	Genus	Species	ITS1	ITS2	*trnL*-P6
Plumbaginaceae	*Limonium*	*vulgare*	2	3	
Polygonaceae	*Fallopia*				2
	Polygonum	*aviculare*	1	1	
	Rumex				4
Ranunculaceae	*Aconitum*		1		
	Anemone				2
		hupehensis	3	3	
	Clematis		1		
	Ranunculus				3
		flammula	2	2	
Rosaceae					22
	Potentilla	*anserina*	4	4	9
	Prunus		1	4	
	Rosa		8	14	
	Rubus		8	3	
	Spiraea		1	2	11
Salicaceae					5
Sapindaceae	*Acer*				1
Scrophulariaceae	*Buddleja*				20
		officinalis	1		
		davidii		13	
Solanaceae					6
	Solanum	*dulcamara*	10	17	
Pinaceae	*Pinus*				25

Taxa are not listed in a hierarchical manner (e.g., *Lotus* and *Lotus pedunculatus* counts originate from different ASVs). Each count represents a taxonomic identification in one bumblebee specimen of 2019. Identifications shown here were made before any read abundance cutoffs were applied to the data.

Appendix 5

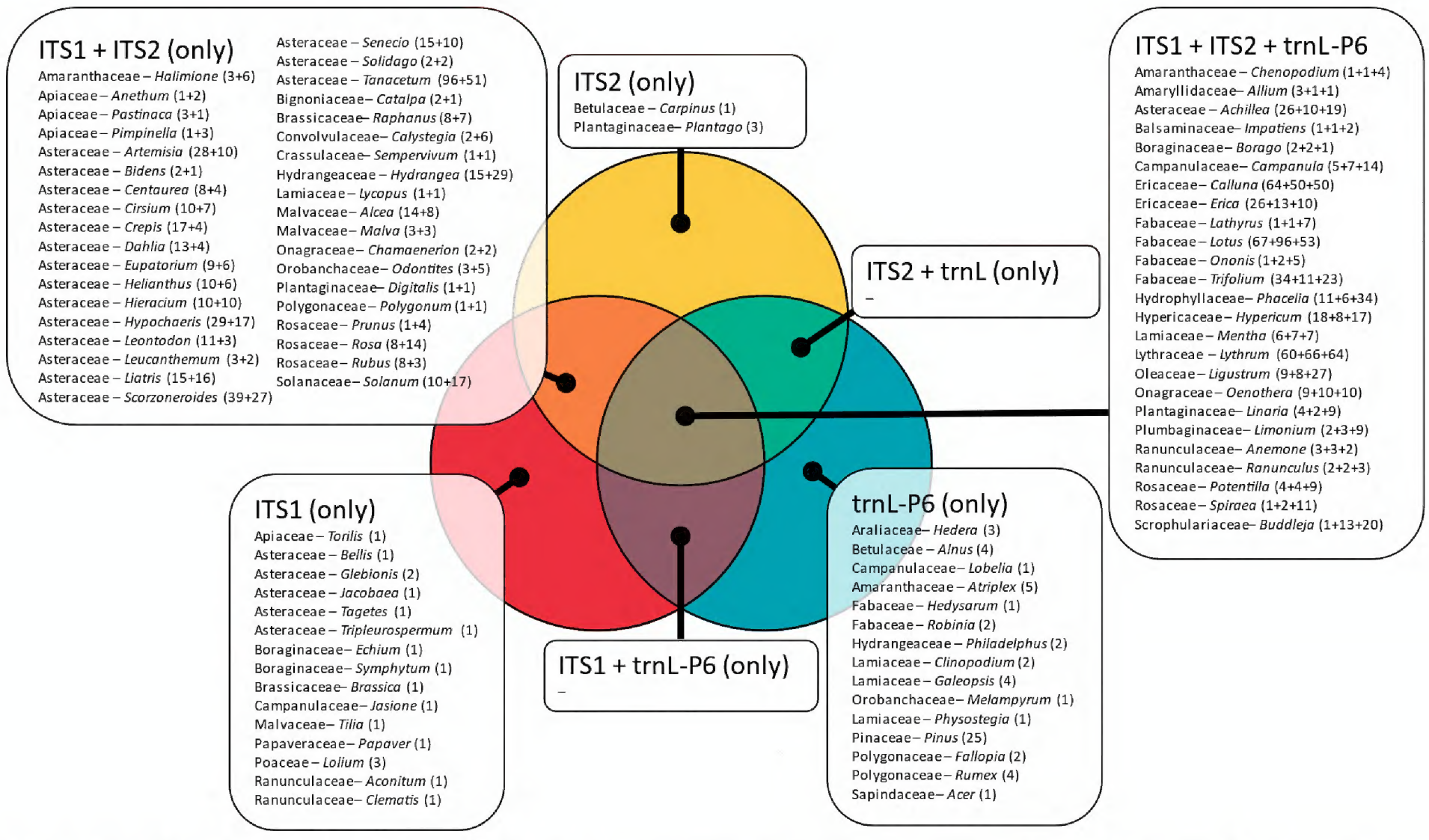

Figure A1. Venn diagram of 2019 marker comparison on genus level. Please note that the large proportion of ITS1+ITS2 (only) detections are due to a lack of resolution in the trnL-P6 marker (compare: Appendix 4). The number in brackets denote the number of plant taxa presence detections (specimen).

Supplementary material 1

Optimizations

Authors: Andreas Kolter

Data type: workflow

Explanation note: Lab protocol (incl PCR) optimizations.

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Link: https://doi.org/10.3897/mbmg.7.86883.suppl1

Supplementary material 2

DNA extraction protocol

Authors: Andreas Kolter

Data type: protocol

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Supplementary material 3

trnL-P6 protocol - primer sequences

Authors: Andreas Kolter

Data type: PCR protocol & primer

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Supplementary material 4

Reference database protocol

Authors: Andreas Kolter

Data type: workflow

Explanation note: Reference database filtering steps.

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Supplementary material 5

Sequence processing workflow

Authors: Andreas Kolter

Data type: workflow

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Supplementary material 6

R pipeline and reference database

Authors: Andreas Kolter

Data type: R script, fasta file

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Link: https://doi.org/10.3897/mbmg.7.86883.suppl6

Supplementary material 7

Raw ASV data

Authors: Andreas Kolter

Data type: ASV table

Explanation note: ASV table and sample number list, bumblebee voucher information.

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